Purification of the Nicotinic Acid Hydroxylase System of *Pseudomonas fluorescens* KB1

By A. L. HUNT*
Department of Biochemistry and Medical Research Council Unit for Research in Cell Metabolism, University of Oxford

(*Received* 28 *August* 1958)

The nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB1 has previously been shown to be an adaptive enzyme which hydroxylates nicotinic acid in the C-6 position with the uptake of one molecule of oxygen for two molecules of nicotinic acid (Hughes, 1955). The reaction initiates the breakdown of nicotinic acid to ammonia and formic, acetic, lactic and malic acid according to the reactions described by Behrman & Stanier (1957). In cell-free extracts nicotinic acid is converted quantitatively into 6-hydroxynicotinic acid and this compound is not further metabolized. The hydroxyl group added is derived from water and not molecular oxygen, indicating that the reaction probably involves the addition of water followed by dehydrogenation with molecular oxygen as hydrogen acceptor (Hunt, Hughes & Lowenstein, 1957, 1958). The enzyme is attached to insoluble particulate components of the cell. This paper is concerned with the purification of the enzyme, together with the components of an associated electron-transport chain, by deoxycholate extraction of the particles, followed by ammonium sulphate fractionation. Preliminary findings of this work have been communicated (Hunt, 1958).

METHODS

Growth of organisms. P. fluorescens KB1 was grown with aeration at 25° in an inorganic salt medium containing KH_2PO_4, 6·8 g., NH_4Cl, 1·0 g., mineral supplement (Behrman & Stanier, 1957), 1·0 ml., $MgSO_4,7H_2O$, 0·5 g., yeast extract (Oxo Ltd.), 0·2 g. and nicotinic acid, 2·5 g. per litre of demineralized water. The solution was adjusted to pH 6·8 with solid NaOH. Subcultures were maintained on slopes containing in addition 2% of agar. For the preparation of large quantities of cells 40 l. batches of medium were inoculated with 4 l. of a logarithmic-phase culture, and growth was allowed to continue under forced aeration in the stainless-steel container of a commercial washing machine (Hoover Ltd.) fitted with a baffle plate and a stainless-steel cover. The airflow was maintained at levels suboptimum for maximum rates of growth. Small quantities of tributyl citrate were added to minimize foaming. Under the conditions used, cell densities reached 3–4 g. wet wt./l. after growth for 10–12 hr. The cells were harvested in a Sharples centrifuge before the onset of the stationary phase. A further 20 g. of nicotinic acid was added before harvesting, and the culture was agitated with a stream of air during the harvesting period. The washed cells were stored at −15° before disintegrating. Cells with optimum activity assumed a deep red colour when frozen at −15°.

Preparation of cell-free extracts. The frozen cell masses were crushed at −20° without the use of abrasive in a Hughes press (Hughes, 1951), modified to accommodate 70 g. of cells; the cylinder measured 4 cm. in diameter. The press was operated by dropping a steel weight of 50 kg. a distance of 1·8 m. down a cylinder mounted above the piston. The crushed cells were homogenized in 2 vol. of ice-cold 0·066 M-sodium potassium phosphate buffer, pH 7·20 (Clarke, 1928), in a glass homogenizer (Potter & Elvehjem, 1936). Approx. 1 mg. of crystalline deoxyribonuclease was added to 500 ml. of the buffer to reduce the viscosity of the homogenate. The mixture was allowed to stand at 0–2° before centrifuging.

Enzyme assays. Oxygen uptake was measured in a conventional Warburg apparatus at 30°. Cups contained 1·0 ml. of 0·066 M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2 M-substrate, enzyme and water to a final volume of 2·0 ml.

Nicotinic acid hydroxylase activity was assayed spectrophotometrically by the reduction of ferricyanide. Cuvettes contained 1·0 ml. of 0·066 M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2 M-potassium nicotinate, 0·3 ml. of 0·1 M-potassium ferricyanide, enzyme and water to a final volume of 3·0 ml. The blank cell contained water instead of substrate. The reaction was started by the addition of enzyme and reduction was followed at 450 mμ in a Carey recording spectrophotometer. The unit of activity is described as the reduction of 1 μmole of ferricyanide in 10 min. at 25°.

Cytochrome oxidase, reduced pyridine nucleotide cytochrome *c* reductase, reduced pyridine nucleotide dehydrogenase and succinoxidase activities were assayed according to procedures described by Mackler & Green (1956).

Spectrophotometric measurements. Cytochrome spectra were obtained in a Carey wavelength-recording spectrophotometer, model 14M-50, with standard 10 mm. quartz cells or 40 mm. Perspex cells fitted with quartz windows. Difference spectra (reduced minus oxidized) were recorded with the reference cell containing enzyme fully oxidized by the addition of a trace of potassium ferricyanide, and the sample cell containing enzyme reduced by the addition of substrate or a crystal of sodium dithionite. Pyridine and cyanide haemochromogens were prepared as follows. The

* 1851 Overseas Scholar. Present address: Department of Chemistry, University of Indiana, Bloomington, Indiana, U.S.A.

enzyme solution was mixed with an equal volume of 0·4N-NaOH and then an equal volume of freshly distilled pyridine or a small quantity of potassium cyanide. The slight precipitate that formed was removed by centrifuging. Difference spectra were recorded as described above. Total haem-protein concentrations were obtained from the above difference spectra with the μmolar extinction coefficients recommended by Chance (1952). Flavin assays were based on the difference spectrum of the enzyme at 460 mμ according to the method of Green, Mii & Kohout (1955).

Chemical estimations. Total N was determined by a micro-Kjeldahl method; the value for total N was multiplied by 6·25 to give an approximate value for protein N. Total iron was estimated with *o*-phenanthroline (Sandell, 1944) after digestion with HNO_3. Samples were well dialysed against water before digestion and contained only trace amounts of phosphorus.

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge, equipped with Baldwin's (1953) modification of the diagonal-schlieren optical system of Philpot (1938). Measurements were made at a rotor speed of 900 rev./sec., and the rotor temperature was usually 26–28°. Sedimentation coefficients were computed by the method of Cecil & Ogston (1948).

Zone electrophoresis. This was performed in a starch column according to the method of Smithies (1955). The purified enzyme was introduced into a 2–3 mm. slit cut in the centre of the block, in the form of a slurry with powdered starch. After electrophoresis the two halves of the block were removed from the carrier, and each was cut longitudinally and then in halves with a tissue slicer. Corresponding portions of the block were each stained to detect enzyme activity, haem components, flavin pigments and protein, as follows. For hydroxylase activity the starch strip was painted with a solution of the standard assay solution, containing nicotinate, ferricyanide and phosphate buffer. The strip was incubated at room temperature for 15 min. and then painted with a dilute solution of $FeCl_3$ in water. Areas of ferricyanide reduced by nicotinate hydroxylase activity appeared as deposits of ferric ferrocyanide. Flavin pigments were detected by examining the strip under ultraviolet irradiation. Haem pigments were detected by painting the strip with a solution of benzidine in acetic acid, and then with hydrogen peroxide, according to Smithies (1955). Protein was detected by staining with a solution of Amido-Black in methanol–acetic acid, also according to the procedure described by Smithies (1955).

RESULTS

Distribution of activity in whole homogenates

Whole homogenates of crushed cells prepared as indicated above were centrifuged at 60 000 *g* for 45 min. in rotor 30 of the Spinco preparative ultracentrifuge. Under these conditions the insoluble cellular material separated into well-defined particulate layers below a clear-yellow supernatant. The particulate residue contained two main constituents, a heavier brown–pink layer and an upper bright-pink layer. After removal of the supernatant, the latter layer could be removed easily into fresh buffer with the aid of a curved spatula. This material was rehomogenized, recentrifuged at 25 000 *g* for 45 min., and resuspended in half the original volume of fresh buffer. The nature of these fractions and the distribution of nicotinic acid hydroxylase activity in the whole extracts will be described fully elsewhere. The resuspended pink upper layer resembled the cell-wall and protoplast-membrane fraction of the cell (cell wall–membrane fraction), and contained the bulk of the hydroxylase activity of the whole extract. Removal of the soluble inactive proteins of the original homogenate resulted in a three- to five-fold purification of nicotinic acid hydroxylase activity in the cell wall–membrane fraction, based both on oxygen uptake and ferricyanide reduction. This fraction also oxidized succinate, malate, lactate and reduced pyridine nucleotides, but 6-hydroxynicotinic acid was not metabolized. The opaque suspensions could be clarified by suspension in a 30 % (w/v) solution of serum albumin (The Armour Laboratories), which allowed spectrophotometric observations to be made. Such suspensions retained the full enzymic activity of the original material. Under these conditions, marked reduction of cytochrome and flavin components could be observed on the addition of nicotinate, succinate or reduced pyridine nucleotides. Thus the cell wall–membrane fraction contains electron-transport chains for the oxidation of these substances, as do mitochondrial systems in higher organisms.

Oxygen could be replaced by ferricyanide, methylene blue and indophenol dyes as electron acceptors in the hydroxylation of nicotinic acid.

Elution of nicotinic acid hydroxylase activity

The cell wall–membrane fraction may be washed repeatedly by centrifuging and resuspension in buffer without loss of activity. However, treatment with buffer containing a detergent released varying amounts of activity into the supernatant. Of reagents tested, sodium deoxycholate proved most effective and, under optimum conditions, released up to 80 % of the bound activity. The effect of detergent treatment is shown in Table 1. Deoxycholate also released considerable quantities of flavin and haem pigments. Both the yield of hydroxylase activity and the latter pigments varied greatly with the ratio of protein to deoxycholate concentration. These relationships are illustrated in Table 2. Increasing deoxycholate released increasing amounts of hydroxylase, flavin and haem pigments up to a ratio of approx. 1 mg. of deoxycholate to 12 mg. of protein. Beyond this level increasing deoxycholate released increasing amounts of flavin pigments but little further hydroxylase or haem pigment.

Nicotinic acid hydroxylase activity in these eluates coupled rapidly with ferricyanide, indophenols and methylene blue as hydrogen acceptors

Table 1. *Effect of detergents on the cell wall–membrane fraction*

Suspensions of the cell wall–membrane fraction (4·0 ml., 162 mg. of N × 6·25) were homogenized in 0·066 M-sodium potassium phosphate buffer, pH 7·20, with detergents as indicated [sorbitan mono-oleate (Tween; Honeywill and Stein Ltd., London); polyethylene glycol complex (Gemex; British Drug Houses Ltd.)]. The homogenates were centrifuged at 60 000 *g* for 45 min. The residues were resuspended in the same volume of fresh buffer. Activities represent total units based on ferricyanide reduction.

	Phosphate	0·3% (w/v) Tween	0·3% (w/v) Gemex	0·3% (w/v) Deoxycholate
Residue	7300	7200	7200	2000
Supernatant	0	400	250	8000

Table 2. *Effect of deoxycholate concentration on the elution of nicotinic acid hydroxylase activity, flavin and haem pigments from the cell wall–membrane fraction*

Suspensions of the cell wall–membrane fraction (8·0 ml., 300 mg. of N × 6·25) were homogenized in 0·066 M-sodium potassium phosphate buffer, pH 7·20, containing varying amounts of sodium deoxycholate as indicated. The homogenates were centrifuged at 60 000 *g* for 45 min. at 0°. Hydroxylase activity, total protein, flavin and haem pigments were assayed in the supernatant. Enzyme activity is expressed as total units of the fraction based on ferricyanide reduction. Figures for haem-protein and flavin are given as μm-moles.

	Concn. of deoxycholate (mg./ml.)			
	0	1·5	3·0	6·0
Cell wall–membrane fraction	4000	—	—	—
Units eluted	0	2000	3200	3600
Protein eluted (mg.)	0	40	61	76
Specific activity of the eluate	—	50	52	47
Total haem eluted	0	5·1	9·6	9·2
Total flavin eluted	0	56·0	80·0	110·0

but not with oxygen. Deoxycholate inhibited oxygen uptake completely in other fractions and could be removed by prolonged dialysis from the above eluates, but this treatment usually resulted in complete loss of hydroxylase activity. The enzyme was similarly unstable in the presence of deoxycholate; activity was lost completely overnight at −15°.

Electron micrographs of the cell wall–membrane fraction before and after extraction are shown in Figs. 1 and 2. Although retaining the shape of the original structure, the treated material was less electron-dense and lay very flat on the grid, indicating that deoxycholate did not disintegrate the fraction but eluted protein and other constituents from further structural material.

Hydroxylase activity may also be released into a supernatant fraction by ultrasonic disruption, but

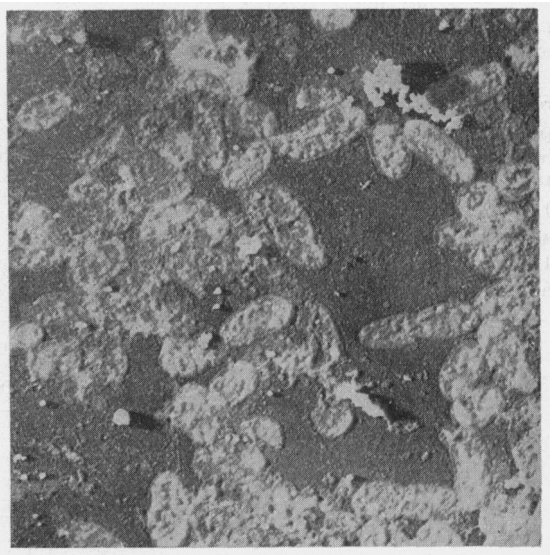

Fig. 1. Electron micrograph of the cell wall–membrane fraction. Shadowed with platinum.

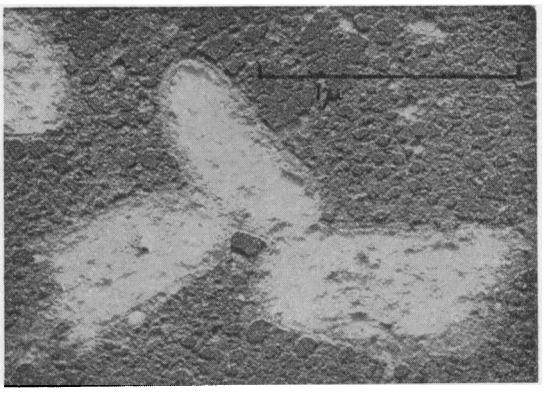

Fig. 2. Electron micrograph of the cell wall–membrane fraction treated with deoxycholate. Shadowed with platinum.

such activity remains attached to polydisperse fragments of the larger particle fraction, and may be sedimented at ultracentrifugal speeds.

Fractionation of deoxycholate extracts

In the analytical ultracentrifuge, deoxycholate extracts showed slow-moving polydisperse components. Whereas hydroxylase activity remained in the supernatant after centrifuging at 60 000 *g* for 60 min., the behaviour of the enzyme in the presence of low concentrations of ammonium sulphate indicated that activity was probably associated with finely divided particulate material. This latter property formed the basis of further purification of the enzyme from deoxycholate extracts. The following procedure was used (Fig. 3).

The washed cell wall–membrane fraction was resuspended in ice-cold 0·066 M-sodium potassium phosphate buffer, pH 7·20 (40–50 mg. of N × 6·25/ml.). The suspension was homogenized in a glass Potter–Elvehjem homogenizer, with the addition of 1·2 % (w/v) deoxycholate solution, previously adjusted to pH 7·20, to give a final concentration of 1·0 mg. of deoxycholate/12·5 mg. of N × 6·25. The homogenate was centrifuged at 60 000 *g* for 45 min. at 0° and the orange–yellow supernatant transferred to a flask immersed in ice. The residue was usually re-extracted in a similar manner and the supernatants were pooled. Nicotinic acid hydroxylase activity was assayed in the supernatant fraction immediately after preparation. Saturated ammonium sulphate solution containing mM-disodium ethylenediaminetetra-acetate, and adjusted to pH 7·20 by glass electrode with aq. NH_3 soln. (sp.gr. 0·880), was then added dropwise with stirring until the concentration reached 15 % saturation. The mixture was allowed to stand for 30 min. at 0°. A bulky white precipitate formed at this stage and was removed by centrifuging at 0°. The latter contained traces of hydroxylase activity and was discarded. Further ammonium sulphate was added to the supernatant to 35 % saturation, and the precipitate recovered by centrifuging at 0°. The deep-yellow supernatant solution was discarded. The precipitate was pink in colour and contained the bulk of the hydroxylase activity of the deoxycholate extract. This material redissolved in fresh buffer to give an optically clear brick-red solution. Hydroxylase activity was assayed in a diluted portion of the solution and the

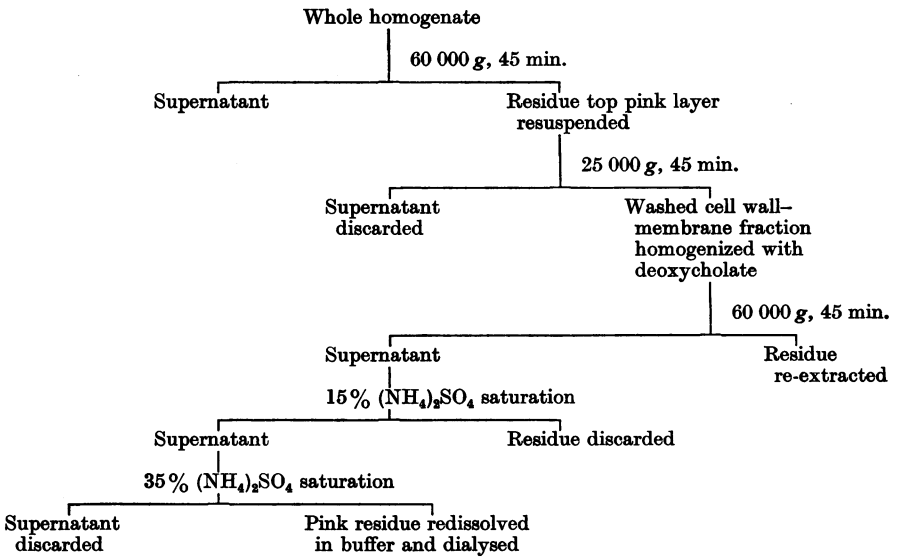

Fig. 3. Purification scheme.

Table 3. *Purification of the nicotinic acid hydroxylase system*

Fraction	10^{-3} × Total units	Protein N × 6·25 (mg.)	Specific activity (units/mg. of N × 6·25)
Whole homogenate	400	32 000	12·5
High-speed supernatant	50	17 500	2·8
Cell wall–membrane fraction	261·5	7 500	35·0
Deoxycholate extract	130	2 000	65·0
15–35 % $(NH_4)_2SO_4$ fraction	90	150	600

remainder dialysed at 0–2° against 1000 vol. of the same buffer for 10–12 hr. Approx. 10% of the activity was lost on dialysis. Most preparations averaged 500–600 units of activity/mg. of protein, based on ferricyanide reduction, representing a 50-fold purification over the original whole extract. The activities at various stages of purification are indicated in Table 3.

Properties of the purified enzyme

The enzyme hydroxylated nicotinic acid rapidly with ferricyanide, indophenol dyes and methylene blue as electron acceptors. In well-dialysed preparations oxygen could also act as the terminal electron acceptor although oxygen uptake was inhibited greatly by trace amounts of deoxycholate. Purification based on oxygen uptake averaged 15-fold. Oxygen uptake was stimulated markedly by the addition of phenazine methosulphate, and was inhibited completely by mM-cyanide and 50% by mM-sodium azide. Cyanide was not inhibitory in the early stages of reduction with ferricyanide as electron acceptor, but it inhibited markedly as the reaction progressed. Inhibition in this case was not enhanced by preincubation with cyanide or with cyanide plus 6-hydroxynicotinic acid.

Nicotinate was the only substrate attacked by the preparation (Table 4). Although the cell wall–membrane fraction oxidized succinate, malate, lactate and reduced pyridine nucleotides, these substrates were not metabolized in the purified preparation. Addition of reduced pyridine nucleotides also failed to reduce the flavin and haem pigments in the fraction. Reduced mammalian cytochrome *c* was not reoxidized, nor was the oxidized compound reduced in the presence of nicotinate. Although a purified cytochrome *c* preparation has been obtained from this organism (Kogut, 1957), no attempt has been made to assay cytochrome reductase or cytochrome oxidase with this compound as donor or acceptor. Oxidative phosphorylation with nicotinate as substrate could not be demonstrated.

Addition of nicotinate in the absence of electron acceptors caused a marked change in the spectrum of the solution. This change was enhanced by the addition of a trace of potassium cyanide and still further by addition of sodium dithionite. The difference spectrum (reduced minus oxidized) showed absorption maxima at 560, 553, 550, 530, 523 and 428 mμ, with a shoulder at 510–515 mμ

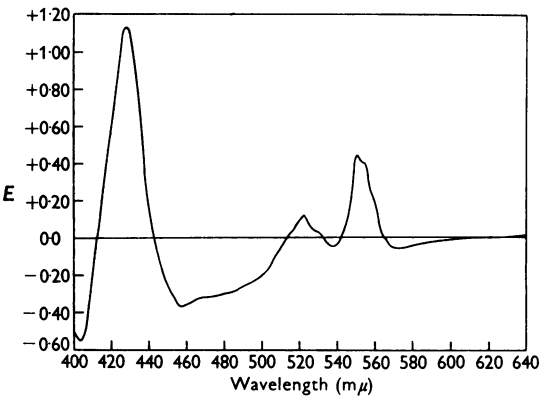

Fig. 4. Difference spectrum (reduced minus oxidized) of the purified nicotinic acid hydroxylase complex, reduced by the addition of sodium dithionite. Cuvettes (40 mm.) contained 3·3 mg. of protein/ml.

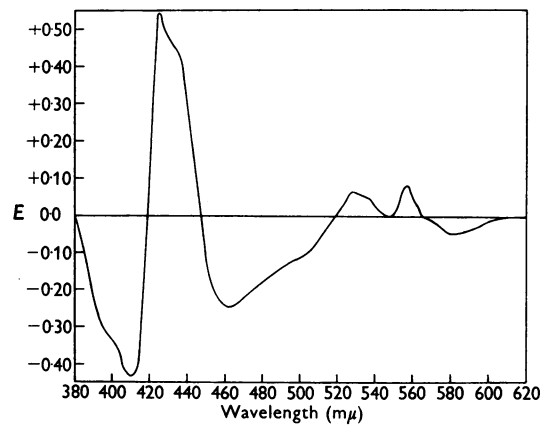

Fig. 5. Difference spectrum (reduced minus oxidized) of the cyanide haemochromogen derivative of the purified nicotinic acid hydroxylase complex.

Table 4. *Reaction of the cell wall–membrane fraction and the purified enzyme with oxygen and phenazine methosulphate*

Warburg cups contained 1·0 ml. of 0·066 M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2 M-substrate, water and 50 mg. of N × 6·25 of cell wall–membrane fraction, 4·2 mg. of N × 6·25 of the purified enzyme and 2·9 mg. of phenazine methosulphate where indicated. Final volume 2·0 ml. Temperature 30°; atmosphere air. Figures represent μmoles of O_2 uptake/hr./mg. of N × 6·25, corrected for blank respiration.

	Nicotinate	Succinate	Malate	Lactate	Nicotinate + phenazine methosulphate	Succinate + phenazine methosulphate
Cell wall–membrane fraction	2·6	1·1	1·3	0·1	—	—
Purified enzyme	34	0	0	0	46	0

and minima at 574, 537 and 460 mμ. A tracing of the difference spectrum recorded on the Carey spectrophotometer is presented in Fig. 4. The reduced complex was rapidly reoxidized by the addition of 0·1 mM-hydrogen peroxide. The difference spectrum of the reduced cyanide haemochromogen derivative of the enzyme preparation exhibited maxima at 556, 537, 526 and 427 mμ, with minima at 547 and 460–467 mμ (Fig. 5). The corresponding pyridine haemochromogen exhibited maxima at 552 mμ, a broad peak between 517 and 522 mμ, and at 415 mμ. After treatment in the dark with pure carbon monoxide, the dithionite-reduced enzyme exhibited maxima not significantly different from those obtained without carbon monoxide treatment. The enzyme contained 1·2 μm-moles of haem and 1·8–2·8 μm-moles of flavin/mg. of N × 6·25, in several preparations. Only half of the flavin was reduced in the presence of nicotinate. The preparation also contained 7 moles of non-haem iron/mole of haem iron, based on *o*-phenanthroline colour.

In the analytical ultracentrifuge, the purified enzyme showed rapidly moving polydisperse components together with slower heterogeneous peaks sedimenting at S_{20} 9·6, 7·3 and 2·0.

The preparation was also subjected to zone electrophoresis in a starch block according to the method of Smithies (1955). Detection of haem, flavin, hydroxylase activity and protein was carried out as indicated under Methods. At pH 8·68 in 0·03 M-sodium potassium phosphate buffer, the enzyme activity, haem components and some of the flavin pigment failed to migrate more than a few millimetres in the starch gel. Three other protein components, one of which was a second flavoprotein, moved in the direction of the cathode. Varying the buffer concentration and the pH of the solution failed to produce any variation in the pattern except in the degree of resolution of the mobile components. It must be assumed therefore that either the enzyme, haem and flavin complex are uncharged at the pH values used, or that the starch granules prevent the movement of the complex in the electrical field.

DISCUSSION

Together with the demonstration that the hydroxyl group added to the C-6 position is derived from water, the above evidence affords evidence for the mechanism of nicotinic acid hydroxylation. This reaction sequence most probably involves addition of water to the ring structure followed by dehydrogenation with oxygen as hydrogen acceptor and takes place through a flavin enzyme and the electron-transport system of the cell. The intermediate steps in the reaction are unknown. Harary (1957), using the chemical analogy of the oxidation of *N*-methylpyridinium hydroxide to *N*-methylpyridone, suggested the reaction involved the addition of the hydroxyl group to the C-2 position, with the formation of the 2-hydroxyl-1:2-dihydro compound, followed by dehydrogenation to the pyridone and rearrangement. It is of interest that the present preparation failed to metabolize the 2-fluoro-, the *N*-methyl- and the 5-fluoro-derivatives of nicotinic acid. Similarly nicotinic acid *N*-oxide was not converted into the 6-hydroxy compound, nor reduced to nicotinic acid in the presence of reduced benzyl-Viologen, suggesting that this compound is not involved in the reaction.

Deoxycholate has been used extensively in the elution of particulate enzymes and in the comminution of larger cell fragments of animal-cell preparations (Green, 1956–57). Many components of the mitochondrial system have been separated with varying concentrations of deoxycholate and salt. In particular, reduced pyridine nucleotide and succinic acid oxidase complexes have been separated and evidence for separate electron-transport chains for these sources described (Rabinowitz & De Bernard, 1957). However, there have been few reports of the use of this reagent for similar purposes in micro-organisms. King & Cheldelin (1957) recently reported the elution of glucose oxidase from subcellular particles of *Acetobacter suboxydans*.

The cytochrome system and oxidative phosphorylation enzymes appear to be localized in the protoplast membrane or an equivalent structure in the bacterial cell. This structure approximates the mitochondrion of animal cells. The present report adds supporting evidence to the concept that organization of the electron-transport system in the bacterial membrane may be a counterpart of that found in the animal mitochondrion. The hydroxylase system, isolated from the structure under the conditions of deoxycholate and salt concentration described, is associated with an electron-transport chain devoid of succinoxidase and reduced pyridine nucleotide oxidase activity. The nature of the purified complex is under further investigation, but preliminary electrophoresis experiments indicate the presence of a complex of haem, flavin and hydroxylase activity, and several other components, one of which is a second flavoprotein.

There have been several reports of the cytochrome components of *Pseudomonas* species. Smith (1955) described absorption maxima at 424, 523, 560 and 580 mμ for whole cells of *P. fluorescens*. Vernon (1956*a*, *b*) described the purification and properties of cytochromes from *Pseudomonas denitrificans* and a further unidentified species. These components were identified as cytochrome *c* with maxima at 550, 520 and 416 mμ, cytochrome c_1 with maxima at 553, 523 and 419 mμ and cytochrome b_1 with maxima at 559, 528 and 426 mμ.

Kogut (1957) also described the purification of the '553 cytochrome' from *P. fluorescens* KB 1. The difference spectrum (reduced minus oxidized) of the present complex also indicates the presence of cytochromes *c*, c_1 and b_1. The combined Soret band, however, is displaced several mμ towards longer wavelengths than would be expected from the combined bands of these components alone. The cyanide and pyridine haemochromogens confirm the identity of these components although the additional absorption at 435 mμ in the cyanide compound does not correspond to these compounds.

No evidence for a typical cytochrome oxidase component could be obtained in this preparation. Chance & Smith (1955) reported the existence of an unusual oxidase component in *Staphylococcus albus*. The reduced compound had absorption maxima at 553 and 424–430 mμ, and reacted with carbon monoxide to give a derivative with maxima at 567, 535 and 415 mμ. Recent studies of Bartsch & Kamen (1958) with the purified compound indicate in addition minima at 485 and 640 mμ, and a Soret maximum at 432 mμ. Although the present reduced complex has maxima at 553 and at 428 mμ, no carbon monoxide derivative could be formed under the conditions used by these workers.

Cytochrome *c* peroxidase has been suggested as a terminal respiratory enzyme in cultures of *P. fluorescens* grown under anaerobic conditions in the presence of nitrate (Lenhoff & Kaplan, 1956). This activity has not been fully investigated in the present complex, but the reduced enzyme is rapidly reoxidized by low concentrations of hydrogen peroxide, suggesting the presence of peroxidase activity. Cytochrome b_1 of *Pseudomonas* is itself autoxidizable but cyanide-insensitive, and the reduced cytochrome c_1 is only very slowly oxidized in air. Thus in the absence of further evidence it must be assumed that terminal respiration in the purified complex is mediated by a peroxidase enzyme of the type described by Lenhoff & Kaplan (1956).

SUMMARY

1. The nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB 1 is associated with a particulate fraction, resembling the cell wall and protoplast membrane of the organism, in homogenates of crushed cells.

2. The enzyme has been eluted by deoxycholate extraction of the particulate fraction and further purified by ammonium sulphate fractionation. Overall purification averaged 50-fold, based on ferricyanide reduction.

3. The purified enzyme hydroxylated nicotinic acid with oxygen, ferricyanide, methylene blue, indophenol dyes and phenazine methosulphate as electron acceptors.

4. Succinate and reduced pyridine nucleotides were not oxidized by the preparation, although these compounds were readily utilized by the parent particulate fraction.

5. The purified preparation contained cytochromes *c*, c_1 and b_1 and flavoprotein components, which were rapidly reduced on the addition of nicotinic acid. Terminal oxidation is tentatively attributed to cytochrome *c* peroxidase activity.

6. The mechanism of nicotinic acid hydroxylation and the organization of the bacterial respiratory chain are discussed.

The author is indebted to Dr D. E. Hughes for assistance in growing micro-organisms, to Dr G. Meek for electron micrographs, to Dr A. Rodgers and Dr A. G. Ogston, F.R.S., for the ultracentrifugal analyses and to Miss S. Graham for able technical assistance. The author also wishes to thank Dr J. Lowenstein for helpful discussions and Professor Sir Hans A. Krebs, F.R.S., for the privilege of working in his Laboratory. This work was assisted by a grant from the Rockefeller Foundation.

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